Simultaneous determination of nucleotide sugars with ion-pair reversed-phase HPLC

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Nucleotide sugars are important in determining cell surface glycoprotein glycosylation, which can modulate cellular properties such as growth and arrest. We have developed a conventional HPLC method for simultaneous determination of nucleotide sugars. A mixture of nucleotide sugars (CMP-NeuAc, UDP-Gal, UDP-Glc, UDP-GalNAc, UDP-GlcNAc, GDP-Man, GDP-Fuc and UDP-GlcUA) and relevant nucleotides were perfectly separated in an optimized ion-pair reversed-phase mode using Inertsil ODS-4 and ODS-3 columns. The newly developed method enabled us to determine the nucleotide sugars in cellular extracts from 1×10^6 cells in a single run. We applied this method to characterize nucleotide sugar levels in breast and pancreatic cancer cell lines and revealed that the abundance of UDP-GlcNAc, UDP-GalNAc, UDP-GlcUA and GDP-Fuc were a cell-type-specific feature. To determine the physiological significance of changes in nucleotide sugar levels, we analyzed their changes by glucose deprivation and found that the determination of nucleotide sugar levels provided us with valuable information with respect to studying the overview of cellular glycosylation status.

Keywords: ion-pair RP-HPLC/nucleotide sugar

Introduction

Glycosylation is regulated by various factors including glycosyltransferases, gene expression, localization and substrate availability including acceptor and donor substrates such as nucleotide sugars. Altered glycosylation profiles of cell surface proteins are one of the hallmarks of malignant cells (Hakomori 1989). Indeed, the expression of N-linked glycans bearing β1-6 GlcNAc-branched and terminal Lewis antigen was elevated in malignant cells associated with progression and metastasis (Fernandes et al. 1991; Dennis et al. 1999). Because these glycan alterations can arise from enhanced activities or elevated expression of glycosyltransferases, the majority of previous studies have focused on the expression of particular glycosylation enzymes (Lowe and Marth 2003; Ohtsubo and Marth 2006; Taniguchi et al. 2006). Very few reports, however, have been published regarding nucleotide sugars, which regulate glycosyltransferase activity and subsequent glycosylation profiles.

The intracellular uridine-5′-diphospho-*N*-acetyl-D-glucosamine (UDP-GlcNAc) level affects branching patterns of N-linked glycans because *N*-acetylglucosaminyl transferases (GnT) have different apparent Km values for the donor substance, UDP-GlcNAc (Oguri et al. 1997; Sasai et al. 2002), which determines the threshold of glycosyltransferase activity in a physiological context. Elevated intracellular UDP-GlcNAc level enhances the activities of GnT-IV and GnT-V, which have higher Km values than other GnTs, increases the number of highly branched N-glycans on growth factor receptors and then turns on the molecular switch promoting cell proliferation (Lau et al. 2007; Stanley. 2007; Taniguchi. 2007). Conversely, low intracellular UDP-GlcNAc levels diminish GnT-IV and GnT-V activities and preferentially and relatively lead to the activation of GnT-III, which has a low Km value, and the formation of highly branched N-glycans by GnT-IV, and GnT-V is suppressed (Yoshimura et al. 1995; Taniguchi et al. 2004; Gu et al. 2009). However, there is still only limited information regarding stoichiometric relevance of nucleotide sugars on glycosylation patterns and subsequent biological events.

Only limited nucleotide sugar species in biological samples have been determined by different approaches such as capillary electrophoresis (Lehmann et al. 2000; Feng et al. 2008), capillary electrophoresis-mass spectrometry (Soo et al. 2004; Soo and Hui 2010), ion-exchange and reversed-phase high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (Coulier et al. 2006). Ion-exchange HPLC is a traditional technique for the determination of nucleotide sugar levels in biological samples. For example, cytidine-5′-monophospho-*N*-acetyl-D-neuraminic acid (CMP-NeuAc) and CMP-NeuGc in the liver and Chinese hamster ovary (CHO) cells can be practically determined by ion-exchange HPLC (Kozutsumi et al. 1990; Terada et al. 1993; Potvin et al. 1995). Uridine-5′-diphospho-D-galactose (UDP-Gal)/uridine-5′-diphospho-D-glucose (UDP-Glc) and uridine-5′-diphospho-*N*-acetyl-D-galactosamine (UDP-GalNAc)/UDP-GlcNAc can

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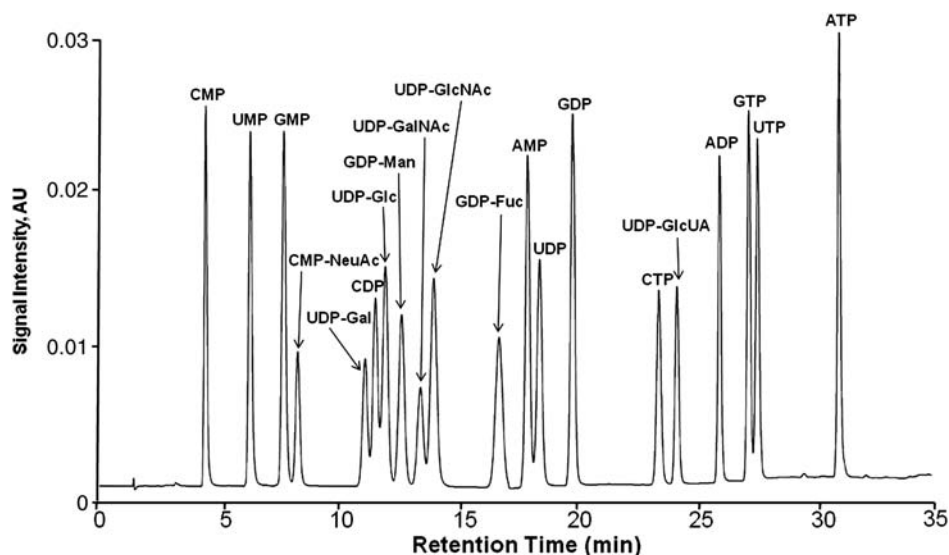
Fig. 1. Separation of a series of nucleotide sugars and nucleotides by optimized ion-pair reversed-phase HPLC on an Inertsil ODS-4 column. An aliquot (20 μl) of a standard mixture (500 pmol each of CMP-NeuAc, UDP-Gal, UDP-Glc, GDP-Man, UDP-GalNAc, UDP-GlcNAc, GDP-Fuc, UDP-GlcUA, AMP, ADP, ATP, CMP, CDP, CTP, UMP, UDP, UTP, GMP, GDP and GTP) was injected onto the column and then eluted as described in the "Materials and methods" section. The eluted compounds were detected by their absorbance at 254 nm.

also be determined using a similar method (Robinson et al. 1995). Simultaneous separation of a series of nucleotide sugars has been achieved by anion-exchange HPLC using a CarboPac PA-1 column. However, co-elution of UDP-GalNAc and adenosine monophosphate (AMP) has been observed in cell samples, making it impossible to determine the level of all nucleotide sugars in cell extracts (Tomiya et al. 2001).

Ion-pair reversed-phase HPLC is a conventional technique, owing to the stability of the silica stationary phase of the column and the nucleotide sugars in the eluent (Ryll and Wagner 1991; Meynial et al. 1995). Simultaneous determination of

five nucleotide sugars (UDP-Gal, UDP-Glc, guanosine-5′-diphosphate-D-mannose (GDP-Man), UDP-GalNAc and UDP-GlcNAc) and eight nucleotides in cell samples has been achieved (Kochanowski et al. 2006). As far as we know, none of the methods has succeeded in mutual separation of individual nucleotides and nucleotide sugars involved in glycosylation.

In the present study, we improved and optimized an ion-pair reversed-phase HPLC method by using an appropriate column for simultaneous separation of a series of nucleotide sugars and nucleotides. We utilized the newly developed method to deter-
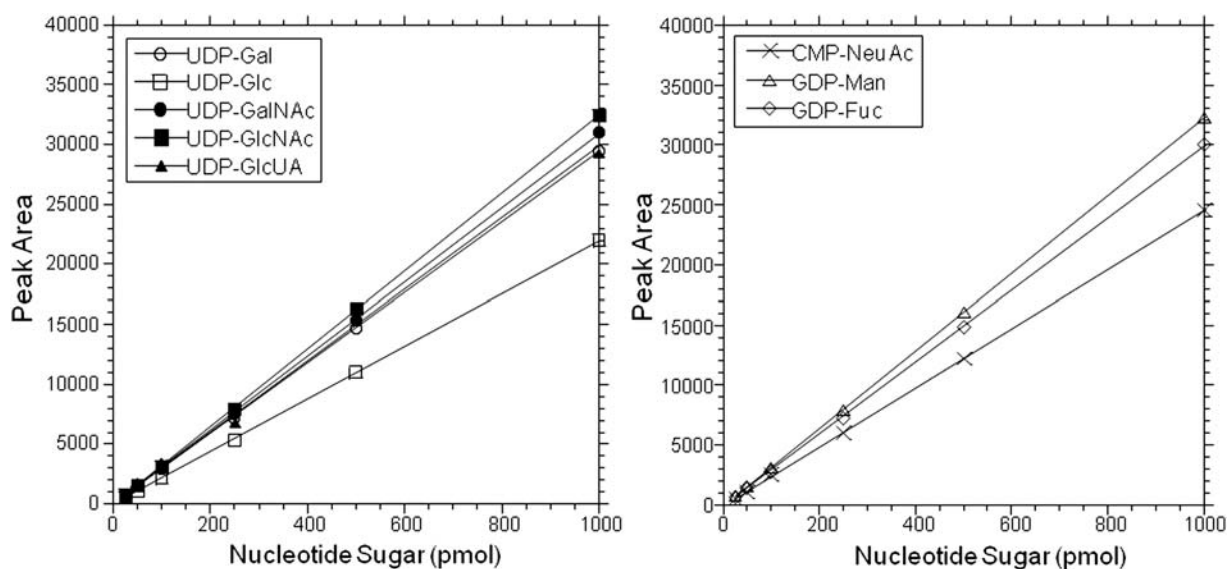

Fig. 2. Calibration curves of a series of nucleotide sugars. A mixture of nucleotide sugars (2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000, 5000 and 10,000 pmol each of CMP-NeuAc, UDP-Gal, UDP-Glc, GDP-Man, UDP-GalNAc, UDP-GlcNAc, GDP-Fuc and UDP-GlcUA) was injected onto the column and then eluted as described in the "Materials and methods" section.

Table I. Reproducibility of elution times and the peak areas of nucleotide sugars

	Elution time (min)			Peak area		
	Mean	SD[a]	CV (%)[b]	Mean	SD	CV (%)
CMP-NeuAc	8.2	0.09	1.1	225,800	9640	4.3
UDP-Gal	11.0	0.12	1.1	262,800	8810	3.4
UDP-Glc	11.8	0.13	1.1	221,200	8100	3.7
GDP-Man	12.5	0.15	1.2	260,700	10,000	3.9
UDP-GalNAc	13.2	0.15	1.1	265,700	5700	2.2
UDP-GlcNAc	13.8	0.16	1.1	265,100	7000	2.7
GDP-Fuc	16.6	0.21	1.3	299,100	12,000	4.0
UDP-GlcUA	24.0	0.13	0.5	272,000	5700	2.1

A mixture of eight nucleotide sugars (about 1 nmol of each) was analyzed by optimized ion-pair RP HPLC.
The data were collected from five HPLC runs.
[a]SD, standard deviation.
[b]CV, coefficient of variance.

mine the nucleotide sugar levels in cancer cell lines and revealed the cell-line-specific changes of nucleotide sugar levels in response to glucose deprivation.

Results

Simultaneous separation of nucleotide sugars by ion-pair reversed-phase HPLC

To simultaneously separate individual nucleotide sugars and nucleotides on an ion-pair reversed-phase HPLC and in particular, to obtain a better separation of UDP-GlcNAc and UDP-GalNAc, we investigated the optimal elution conditions using a mixture of eight nucleotide sugars (CMP-NeuAc, UDP-Gal, UDP-Glc, GDP-Man, UDP-GalNAc, UDP-GlcNAc, guanosine-5′-diphosphate-L-fucose (GDP-Fuc) and uridine-5′-diphospho-D-glucuronic acid (UDP-GlcUA)) and 12 nucleotides (AMP, adenosine diphosphate (ADP), adenosine triphosphate (ATP), cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), guanosine monophosphate (GMP), guanosine diphosphate (GDP) and guanosine triphosphate (GTP)). An optimal elution buffer was reported in a recently reported method (Kochanowski et al. 2006). In the present study, we selected an appropriate column from five types of reversed-phase columns with different stationary phase characteristics in their silica particles, since the stationary phase characteristics on a reversed-phase column affects the retention of compounds bound to ion-pair agents in an elution buffer Grune and Siems (1993). The use of Inertsil ODS-4 and ODS-3 resulted in good separation of UDP-GlcNAc and UDP-GalNAc (Supplementary Figure 1) as well as individual nucleotide sugars and nucleotides. A single chromatographic step using an optimized elution gradient condition perfectly separated the eight nucleotide sugars and 12 nucleotides (Figure 1).

Linearity, sensitivity and reproducibility

In our new method, the linear range between the injected amount of each nucleotide sugar and the peak area was 2 pmol to 10 nmol (Figure 2). The new method was 7.5-fold more sensitive than those published in previous reports (Meynial et al. 1995; Kochanowski et al. 2006). The detection limit of the nucleotide sugars was 2 pmol/injection. Analytical data of elution times and peak areas of the eight nucleotide sugars were highly reproducible over the five experiments (Table I).

Simultaneous separation of nucleotide sugars in cellular extracts

Nucleotide sugars in cells were extracted with 70% (v/v) ice-cold ethanol followed by solid-phase extraction to ensure efficient collection of unstable CMP-NeuAc (Rabina
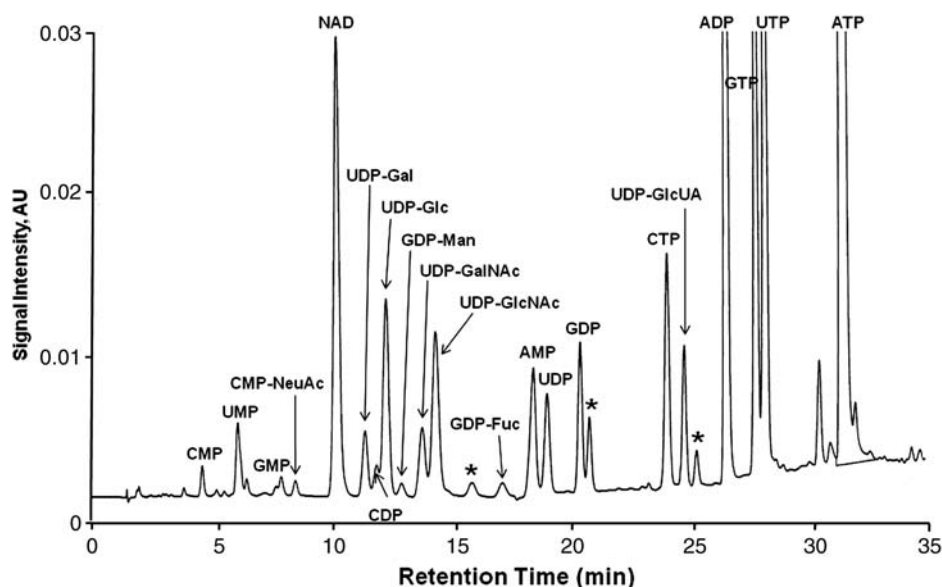

Fig. 3. Separation of nucleotide sugars and nucleotides in CHO cell extracts. CHO cells (2×10^6 cells) were lysed in ice-cold 70% ethanol, and the supernatant fraction was further purified by ion-pair solid-phase extraction. A freeze-dried sample was dissolved in water (100 µl), and an aliquot (20 µl) was injected onto the column. Each peak was identified by comparison with the retention times of a standard mixture. Peaks shown with asterisk were unknown peaks. The nucleotide sugar levels were determined based on the peak areas of calibration curves of the standards and normalized to units of pmol/mg protein. Data are presented in Table II as the means ± SD of triplicate experiments.

Table II. The levels of nucleotide sugars in CHO, MCF7 and KLMl, cultured under normal or glucose-deprived conditions

Nucleotide sugars (pmol/mg protein)

	CMP-NeuAc	UDP-Gal	UDP-Glc	GDP-Man	UDP-GalNAc	UDP-GlcNAc	GDP-Fuc	UDP-GlcUA
Normal								
CHO	271 ± 11	477 ± 57	2124 ± 223	67 ± 12	805 ± 24	1760 ± 5	107 ± 0	422 ± 21
MCF7	166 ± 40	555 ± 21	2059 ± 39	133 ± 16	4904 ± 310	12,420 ± 939	204 ± 5	3922 ± 234
KLM1	380 ± 65	500 ± 88	1757 ± 110	128 ± 15	2088 ± 336	5991 ± 672	170 ± 10	3339 ± 252
MCF7/CHO (-fold)	0.6	1.2	1.0	2.0	6.1	7.1	1.9	9.3
KLM1/CHO (-fold)	1.4	1.1	0.8	1.9	2.6	3.4	1.6	7.9
Glucose deprivation (GD)								
MCF7	172 ± 20	592 ± 15	2317 ± 92	135 ± 31	2542 ± 123	6950 ± 661	259 ± 11	2282 ± 99
GD/normal (-fold)	1.04	1.07	1.12	1.02	0.52	0.55	1.27	0.58
KLM1	304 ± 20	61 ± 22	154 ± 32	7	798 ± 50	1984 ± 104	226 ± 11	700 ± 66
GD/normal (-fold)	0.80	0.12	0.08	0.05	0.38	0.33	1.30	0.21

The data under normal condition are presented as the means ± SD of triplicate experiments, and those under conditions by glucose deprivation (GD) were collected duplicate experiments. The ratios of nucleotide sugar levels in MCF7 and KLM1 for CHO are shown in the middle. The ratios of those under GD conditions for normal are also shown at the bottom, respectively.

et al. 2001; Tomiya et al. 2001; Turnock and Ferguson 2007). To validate the feasibility of the extraction method, 2 pmol of guanosine-5′-diphosphate-D-glucose (GDP-Glc), an unnatural nucleotide sugar, was added to the cell pellets before extraction. The recovery of GDP-Glc was found to be greater than 85% ($n = 2$). The recovery of unstable CMP-NeuAc was also approximately $70 \pm 3.8\%$ ($n = 3$) when exogenous CMP-NeuAc was added. Figure 3 shows an elution pattern of the nucleotide sugars and nucleotides from CHO cells. The eight nucleotide sugars were successfully separated from nucleotides as well as non-nucleotide contaminants, such as nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate, and determined based on the peak areas.

The calculated values shown in Table II were similar to those previously described (Tomiya et al. 2001), with the exception of UDP-GalNAc and UDP-GlcUA, which had been not determined in the previous report. The UDP-GlcNAc: UDP-GalNAc and UDP-GlcNAc:UDP-GlcUA ratios were found to be approximately 1:0.45 and 1:0.24, respectively, and quite similar to previously reported values (Sweeney et al. 1993).

Simultaneous determination of nucleotide sugar levels in breast and pancreatic cancer cell lines cultured under normal or glucose-deprived conditions

We focused on the UDP-GlcNAc level, which regulates β1-6 GlcNAc-branch formation, and determined the nucleotide sugars in a breast cancer cell line, MCF7, and a pancreatic cell line, KLM1, both of which were expected to have high levels of UDP-GlcNAc, since they express β1-6 GlcNAc-branched *N*-glycans (Fernandes et al. 1991; Nan et al. 1998). For each cell sample, three separate preparations were analyzed.

In both MCF7 and KLM1 cells, the levels of UDP-GalNAc, UDP-GlcNAc, UDP-GlcUA and GDP-Fuc were significantly higher than those observed in CHO cells (Table II). The levels of UDP-GalNAc, UDP-GlcNAc and UDP-GlcUA in KLM1 were 58%, 52% and 15% lower than those in MCF7, respectively. In contrast, other nucleotide sugars such as CMP-NeuAc were 2.3-fold higher than those in MCF7, and the levels of GDP-Man and GDP-Fuc were similar to the levels observed in MCF7 cells.

Glucose deprivation is one of the important key factors to which tumors must respond and adapt. In order to elucidate the physiological significance of the alterations in nucleotide sugar levels and to examine the effects of nutritional states in culture medium on nucleotide sugar levels, we cultured cells in glucose-deprived medium and determined the nucleotide sugars (Table II). The levels of UDP-GalNAc, UDP-GlcNAc and UDP-GlcUA were significantly reduced in glucose-deprived medium. Interestingly, the levels of UDP-Gal, UDP-Glc and GDP-Man in KLM1 were also remarkably decreased by 88%, 93% and 95%, respectively. Glucose deprivation did not affect the CMP-NeuAc and GDP-Fuc levels in both cell lines. These data indicate that the developed method was effective in the simultaneous determination of a series of nucleotide sugars in cell lines and demonstrated the changes of nucleotide sugar levels in response to altered physiological conditions.

Discussion

In the present study, we developed a HPLC method for simultaneously separating nucleotide sugars by choosing a reversed-phase column in an optimized ion-pair reversed-phase mode. The new HPLC method allowed us, for the first time, to separate not only each nucleotide sugar involved in N- and O-glycan synthesis but also a series of nucleotides, contrary to most of the previously reported methods (Ryll and Wagner 1991; Meynial et al. 1995; Kochanowski et al. 2006; Turnock and Ferguson 2007). The use of Inertsil ODS-4 and ODS-3 was crucial for achieving this separation, particularly that of UDP-GlcNAc and UDP-GalNAc. These columns are end-capped reversed-phase columns with higher hydrophobicity (surface area = 450 m^2/g) than a conventional column (Supplementary Figure 1), and hence, the specific hydrophobicity of the columns appears to be crucial for discriminating the subtle structural differences between UDP-GalNAc and UDP-GlcNAc. Nucleotide sugars can also be eluted under 100% aqueous eluents using ODS-4 and results in high-resolution separation. Furthermore, using a long column of high pressure type (250 × 4.6 mm) allowed complete separation of the nucleotide sugars (Supplementary Figure 2). Since there were no overlapping peaks of nucleotide sugars with major contaminants derived from cells, simultaneous determination of nucleotide sugars in cell samples has been achieved without prior isolation of the nucleotide sugars. The extraction method employed, using ice-cold ethanol followed by solid-phase extraction, resulted in reproducible and comprehensive determination of nucleotide sugar levels. At present, since we could not rule out that some peaks that we observed contain some contaminants in various samples, we are also analyzing the same samples by liquid chromatography-electrospray ionization-mass spectrometry method in an ion-pair reversed-phase mode, which we are developing.

We applied this method to determine nucleotide sugars in breast and pancreatic cancer cell lines. We could detect the elevated UDP-GlcNAc and UDP-GalNAc levels in both cell lines. The high levels of UDP-GlcNAc and UDP-GalNAc in breast tumor tissues has been reported before (Gribbestad et al. 1994), and this high levels in cancer cell lines may be caused by increased glucose uptake because overexpression of glucose transporter 1 has been observed in human breast cancer cell lines (Younes et al. 1996). As for GDP-Fuc, higher levels in both cell lines were observed compared to CHO cells. It has been reported that the high level of GDP-Fuc in cancer cell lines occurs through the elevation of GDP-keto-6-deoxymannose 3,5 epimerase-4-reductase, an enzyme that catalyzes the second step in GDP-Fuc synthesis from GDP-Man (Noda et al., 2002, Noda et al., 2003). For CMP-NeuAc, however, further studies will be required to explain the difference between the two cell lines.

We also observed a decrease in UDP-sugar and GDP-Man levels in a cell-type-specific manner with no change in CMP-NeuAc and GDP-Fuc levels when cultures were glucose deprived (Table II). GDP-Fuc and CMP-NeuAc are synthesized late during de novo synthesis in glucose metabolism (Varki et al. 2008), and thus glucose deprivation did not alter their levels. In glucose-deprived cultures, glucose is supplied from intracellular glycogen through glycogenolysis, and thus the degree of decrease by glucose deprivation may depend on the amount of intracellular storage of glycogen. We unexpectedly observed a remarkable decrease in UDP-Gal, UDP-Glc and GDP-Man levels in KLM1 cells when they were glucose deprived. UDP-Glc and GDP-Man are used for synthesizing dolicol-P-glucose, an N-glycan precursor (Varki et al. 2008), and its intracellular abundance should control N-glycan synthesis. UDP-Gal is a donor in the synthesis of complex-type N-glycans and polylactosamine residues. Therefore, the decrease in these nucleotide sugars in KLM1 cells should alter the amounts and structures of glycans on cell surfaces. Recent work has indicated that glucose deprivation regulates O-GlcNAcylation of cytoplasmic proteins such as transcriptional factors via changes in intracellular UDP-GlcNAc levels (Hart et al. 2007; Taylor et al. 2008). Transcriptional regulation by O-GlcNAcylation may be another factor that results in the remarkable decrease in UDP-Gal, UDP-Glc and GDP-Man mentioned above because it has a potential for transcriptional regulation of the enzymes involved in glucose metabolism (Kang et al. 2009). Determination of the key enzymes involved in decreased nucleotide sugar synthesis is one of our future projects.

In the present study, we demonstrated that our optimized HPLC method is efficient and convenient for simultaneous determination of nucleotide sugar levels and that it is suitable for monitoring cellular changes of donor substances for glycosylation. Our new method provides valuable information for better understanding the glycan profile and subsequent cellular responses.

Materials and methods

Materials

UDP-Gal, UDP-Glc, UDP-GalNAc, UDP-GlcNAc, UDP-GlcUA, CMP-NeuAc and various nucleotides were purchased from Sigma Aldrich Japan (Tokyo, Japan). GDP-Fuc and GDP-Man were purchased from Calbiochem (San Diego, CA). The sources of other materials used in this study were as follows: Dulbecco's modified Eagle's medium (DMEM) with high glucose (Sigma, St Louis, MO); fetal bovine serum (FBS), glucose-free DMEM, penicillin and streptomycin sodium (Invitrogen, Carlsbad, CA); all other chemicals (Wako Chemicals, Osaka, Japan).

Cells and culture

CHO cells and MCF7 were obtained from the ATCC (Manassas, VA). Human pancreatic cancer cell line KLM1 was obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University, Japan). These cells (2.5 × 10^5 cells) were cultured in 6 cm culture dishes in a humidified atmosphere containing 5% CO_2. CHO, MCF7 and KLM1 cells were cultured in DMEM supplemented with 10% FBS and 100 U/ml penicillin and streptomycin for 2-3 days until they reached approximately 80% confluence. For glucose deprivation, each cell line (2.5 × 10^5 cells) was cultured for 2 days under normal conditions, washed once with phosphate-buffered saline (PBS) and then incubated for 48 h in glucose-free DMEM supplemented with 10% FBS and 100 U/ml penicillin and streptomycin.

Preparation of cellular extracts

Cells were washed once with cold PBS, collected in 500 µl cold PBS and spiked with the unnatural nucleotide sugar,

GDP-Glc, as an internal standard to normalize recovery in the preparation. Ice-cold ethanol (1.5 ml) was added to the samples, and cells were lysed using a Handy Sonic Disruptor (Tomy Seiko Co. Ltd., Tokyo, Japan) as previously described (Tomiya et al. 2001). The extract was centrifuged at $16,000 \times g$ for 10 min at 4°C in order to remove insoluble material, and the supernatant was lyophilized. The freeze-dried sample was subjected to ion-pair solid-phase extraction using an Envi-Carb column (Supelco Inc, Bellafonte, PA) conditioned with 80% acetonitrile in 0.1% trifluoroacetic acid (3 ml) followed by 2 ml of water, as previously reported (Rabina et al. 2001). Briefly, each sample was dissolved in 2 ml of 10 mM NH_4HCO_3 and applied to the column. The column was sequentially washed with 2 ml of water, 2 ml of 25% acetonitrile, 200 µl of water and 2 ml of 50 mM triethylamine acetate buffer (pH 7). Nucleotide sugars were eluted with 2 ml of 25% acetonitrile in 50 mM triethylamine acetate buffer (pH 7). The eluate was lyophilized and stored at −80°C until analyzed.

Protein contents

Portions of the cells were lysed in 500 µl water by sonication and centrifuged at $16,000 \times g$ for 10 min. The protein contents in the supernatants were determined using a BCA assay kit (Pierce, Rockford, IL).

Ion-pair reversed-phase HPLC

A Prominence HPLC system (Shimadzu, Kyoto, Japan) was used. Nucleotide sugars were detected by their absorbance at 254 nm. Separation of nucleotide sugars was performed at 40 °C on an Inertsil ODS-4 column (particle size = 3 µm, 150 × 4.6 mm internal diameter; GL Science) in combination with a guard column (particle size = 3 µm; 10 × 4.6 mm). An Inertsil ODS-3 column of the same size (GL Science) was also used. Inertsil ODS-SP, Inertsil ODS-P (GL Science) and Develosil C30-UG-5 (Nomura Kagaku, Aichi, Japan) columns of the same size were used for comparison. A long column of high pressure type (250 × 4.6 mm) was also used for complete separation. For HPLC analysis, buffer A (100 mM potassium phosphate buffer pH 6.4 supplemented with 8 mM tetrabutylammonium hydrogensulphate as an ion-pair reagent) was made using high-purity water (Kanto Kagaku, Tokyo, Japan) and filtered through a 0.45-µm membrane filter before use. Buffer B was 70% buffer A with 30% acetonitrile. Each cellular extract was dissolved in water, and a 20-µl aliquot was injected onto the column equilibrated with buffer A. The elution gradient was as follows: 100% buffer A for 13 min; 0–77% linear gradient of buffer B for 22 min; 77–100% buffer B for 1 min; and 100% buffer B for 14 min. The flow rate was maintained at 1 ml/min. Elution in the long column was performed using the following elution gradient: 100% buffer A for 35 min; 0–77% linear gradient of buffer B for 40 min; 77–100% buffer B for 1 min; and 100% buffer B for 14 min. The flow rate was maintained at 0.8 ml/min.

Peaks were identified by comparison with the retention times of a standard mixture injected before the analysis. Each nucleotide sugar peak was integrated and quantified based on the peak areas of the calibration curve of each standard. Nucleotide sugar levels in cell extracts were normalized from the

recovery of exogenous GDP-Glc and then expressed as pmol/mg protein.

After a set of experiments, the column was washed with 50% acetonitrile/100 mM phosphate buffer without ion-pair agents for 20 min and then 50% acetonitrile/water for 20 min and stored in solution. In order to prevent deterioration of the separation column, the guard column was regularly exchanged when we observed a decrease in peak resolution.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

Abbreviations

ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CDP, cytidine diphosphate; CHO, Chinese hamster ovary; CMP, cytidine monophosphate; CMP-NeuAc, cytidine-5′-monophospho-N-acetyl-D-neuraminic acid; CTP, cytidine triphosphate; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GDP, guanosine diphosphate; GDP-Fuc, guanosine-5′-diphosphate-L-fucose; GDP-Man, guanosine-5′-diphosphate-D-mannose; GDP-Glc, guanosine-5′-diphosphate-D-glucose; GMP, guanosine monophosphate; GTP, guanosine triphosphate; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; UDP, uridine diphosphate; UDP-Gal, uridine-5′-diphospho-D-galactose; UDP-GalNAc, uridine-5′-diphospho-N-acetyl-D-galactosamine; UDP-Glc, uridine-5′-diphospho-D-glucose; UDP-GlcNAc, uridine-5′-diphospho-N-acetyl-D-glucosamine; UDP-GlcUA, uridine-5′-diphospho-D-glucuronic acid; UMP, uridine monophosphate; UTP, uridine triphosphate.

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